Vol. 126, No. 1 February 1964

THE
BIOLOGICAL BULLETIN

PUBLISHED BY THE MARINE BIOLOGICAL LABORATORY

THE EFFECT OF ANTISERA AGAINST FERTILIZIN ON THE UPTAKE OF ORTHOPHOSPHATE BY SEA URCHIN EGGS[1]

JOHN W. BROOKBANK[2]

Department of Microbiology, College of Medicine, and the Department of Biology, University of Florida, Gainesville, Florida

Antisera against fertilizin have been shown to inhibit the cleavage of the zygote (Tyler and Brookbank, 1956a, 1956b) and to increase the respiratory rate of unfertilized and fertilized eggs of some species of sea urchins (Brookbank, 1959). It has been claimed that antisera against egg homogenates or purified fertilizin are parthenogenic (Perlmann, 1957, 1959). These observations have been re-examined and discussed by Brookbank (1959), Tyler (1959), Tyler *et al.* (1961) and Tyler (1963). No clear evidence of egg activation in the presence of such antisera was found by these authors.

The present report deals with the effect on phosphate uptake of antisera against fertilizin, a process known to increase markedly after fertilization and artificial parthenogenesis (see Whiteley and Chambers, 1960; Litchfield and Whiteley, 1959, for data and earlier references). Phosphate uptake by unfertilized eggs is essentially zero. Further, the transport mechanism is presumed to differentiate at the surface of the egg following activation (Whiteley and Chambers, 1960), and therefore should be available for possible interaction with antibody.

Materials and Methods

Eggs and sperm of *Arbacia punctulata* and *Lytechinus variegatus* were obtained by electric shock (Harvey, 1956) or by KCl injection (Palmer, 1937; Tyler, 1949), respectively. Fertilization membranes of *Arbacia* eggs were eliminated by trypsin (1 mg.% for 10 minutes) treatment of unfertilized eggs. Passage of fertilized (1–2 minutes) *Lytechinus* eggs through an 18-gauge needle fixed to a 50-ml. syringe removed the fertilization membranes from most (90%) of these eggs. Fertilizability of the eggs in dilute sperm suspensions was tested after four washes

[1] This research was supported in part by a grant (GM 04659) from the National Institutes of Health.

[2] Author's current address: Department of Biology, University of Florida, Gainesville, Florida. The author wishes to gratefully acknowledge the technical assistance of Janet G. Yonge.

in sea water, before the eggs were used. Only those of 95–100% fertilizability were used.

Antisera were prepared in rabbits against purified fertilizin, as previously described (Brookbank, 1959). All were sea-water-dialyzed, and all were effective in cleavage inhibition. The antisera against *Lytechinus* fertilizin (4 prime and 10 prime) were the same as those used to determine the effects of such antisera on unfertilized egg respiration (Brookbank, 1959). The antisera against *Arbacia* fertilizin (6 prime and 8 prime) have not yet been tested for respiratory effects. Additional antisera were prepared in rabbits against unfertilized eggs of *Lytechinus* (fertilizin included). Pre-injection sera of all rabbits except 4 and 10 were available for use as controls. Control sera for 4 prime and 10 prime antisera were obtained by bleedings of uninjected animals (abbreviated Th., F., and M_1.).

Phosphate uptake was measured using carrier-free P^{32} (Union Carbide Nuclear Corporation) in sea water. The amount of serum available did not permit the use of the perfusion chamber method of Whiteley and Chambers (1960). In the following experiments, unfertilized or fertilized eggs (0.25 ml. of a 25% or 50% suspension) were placed, in duplicate, in 2-ml. screw-cap vials, together with P^{32}-sea water (50 or 100 $mm.^3$) and serum (0.25 ml.). Sea water controls without serum were included. Fertilized eggs were placed in the vials 30 minutes after fertilization

TABLE I

The effect of normal and immune sera on uptake of P^{32} by fertilized eggs (Figures given are averages of duplicate samples; *, 50% ammonium sulfate fraction; L.v., Lytechinus variagatus; A.p., Arbacia punctulata; I, immune; N, normal; C, sea water control; cpm, counts per minute.)*

Experiment	% Egg concentration	Sera used		Ave. % recovery of P^{32}	Cpm eggs	Cpm 100 $mm.^3$ supernatant	Total cpm added to sample
1. *L.v.*	50	4 prime	I	95	63	1815	10,400
		Th.	N		136	1755	
		C			368	1714	
2. *L.v.*	50	4 prime	I	94	92	1771	10,634
		Th.	N		341	1811	
		C			518	1655	
3. *L.v.*	50	4 prime	I	95	506	2306	14,635
		Th.	N		1975	1934	
		C			5895	1338	
4. *L.v.*	50	4 prime	I	100	229	1280	7,317
		Th.	N		570	1227	
		C			3576	704	
5. *L.v.*	25	4 prime	I	89	150	1120	7,201
		Th.	N		472	1136	
		C			1297	936	
6. *L.v.*	25	10 prime	I	92	90	1183	7,320
		F	N		597	1135	
		C			1988	896	

TABLE I—(*Continued*)

Experiment	% Egg concentration	Sera used	Ave. % recovery of P^{32}	Cpm eggs	Cpm 100 mm.³ supernatant	Total cpm added to sample
7. *L.v.*	25	4 prime I	95	185	1157	6,938
		M_1 N		266	1195	
		C		3257	566	
8. *L.v.*	25	4 prime I*	91	138	796	4,944
		M_1 N*		148	776	
		C		262	784	
9. *L.v.*	25	a prime I	95	389	1423	9,495
		a N		968	1428	
		C		1198	1282	
10. *A.p.*	25	8 prime I	97	116	1104	12,922
		8 N		263	1436	
		C		652	1442	
11. *A.p.*	25	6 prime I	95	653	1488	9,900
		6 prime (0.45/1) I		1458	1454	
		6 N*		1282	1462	
		C		3360	835	

* Serum code:

4 prime—anti-*Lytechinus* fertilizin
10 prime—anti-*Lytechinus* fertilizin
a prime—anti-*Lytechinus* fertilizin plus unfertilized eggs
6 prime—anti-*Arbacia* fertilizin
8 prime—anti-*Arbacia* fertilizin
6 —corresponding pre-injection sera
8 —corresponding pre-injection sera
a —corresponding pre-injection sera
Th. —normal sera from 3 uninjected rabbits
F. —normal sera from 3 uninjected rabbits
M_1 —normal sera from 3 uninjected rabbits

in order to by-pass the lag and transition phases of phosphate uptake. The length of the lag period varies from female to female within a species (Whiteley and Chambers, 1960). These authors state that an average lag of 15 minutes in P^{32} uptake occurs following fertilization in *Strongylocentrotus purpuratus*. A count of 50 or 100 mm.³ of the P^{32}-sea water served to indicate total radioactivity at zero time. After incubating with slow rotation at 1 rpm for 60 minutes at 25° C., the vials were centrifuged and 100 mm.³-aliquots of the supernatant taken. The eggs were washed three times in 2-ml. volumes of cold (0° C.) sea water, quantitatively transferred to planchets, and dried. Some difficulty was experienced in transfer of fertilized, demembranated eggs, in that these eggs sometimes tended to stick to glass. Percentage recovery of radioactivity was calculated after counting the dried samples with a thin-end-window Geiger-Muller tube (1.8 mg./cm.²) within a low background (17 cpm) shield. The results are expressed as counts per minute per 0.25 ml. egg suspension, or per 100 mm.³ supernatant sea water. Experiments with 90% recovery or better are included. Comparisons made between experiments are of necessity qualitative since egg suspensions were prepared on the

in sea water, before the eggs were used. Only those of 95–100% fertilizability were used.

Antisera were prepared in rabbits against purified fertilizin, as previously described (Brookbank, 1959). All were sea-water-dialyzed, and all were effective in cleavage inhibition. The antisera against *Lytechinus* fertilizin (4 prime and 10 prime) were the same as those used to determine the effects of such antisera on unfertilized egg respiration (Brookbank, 1959). The antisera against *Arbacia* fertilizin (6 prime and 8 prime) have not yet been tested for respiratory effects. Additional antisera were prepared in rabbits against unfertilized eggs of *Lytechinus* (fertilizin included). Pre-injection sera of all rabbits except 4 and 10 were available for use as controls. Control sera for 4 prime and 10 prime antisera were obtained by bleedings of uninjected animals (abbreviated Th., F., and M_1.).

Phosphate uptake was measured using carrier-free P^{32} (Union Carbide Nuclear Corporation) in sea water. The amount of serum available did not permit the use of the perfusion chamber method of Whiteley and Chambers (1960). In the following experiments, unfertilized or fertilized eggs (0.25 ml. of a 25% or 50% suspension) were placed, in duplicate, in 2-ml. screw-cap vials, together with P^{32}-sea water (50 or 100 $mm.^3$) and serum (0.25 ml.). Sea water controls without serum were included. Fertilized eggs were placed in the vials 30 minutes after fertilization

TABLE 1

The effect of normal and immune sera on uptake of P^{32} by fertilized eggs (Figures given are averages of duplicate samples; *, 50% ammonium sulfate fraction; L.v., Lytechinus variagatus; A.p., Arbacia punctulata; I, immune; N, normal; C, sea water control; cpm, counts per minute.)*

Experiment	% Egg concentration	Sera used		Ave. % recovery of P^{32}	Cpm eggs	Cpm 100 $mm.^3$ supernatant	Total cpm added to sample
1. *L.v.*	50	4 prime	I	95	63	1815	10,400
		Th.	N		136	1755	
		C			368	1714	
2. *L.v.*	50	4 prime	I	94	92	1771	10,634
		Th.	N		341	1811	
		C			518	1655	
3. *L.v.*	50	4 prime	I	95	506	2306	14,635
		Th.	N		1975	1934	
		C			5895	1338	
4. *L.v.*	50	4 prime	I	100	229	1280	7,317
		Th.	N		570	1227	
		C			3576	704	
5. *L.v.*	25	4 prime	I	89	150	1120	7,201
		Th.	N		472	1136	
		C			1297	936	
6. *L.v.*	25	10 prime	I	92	90	1183	7,320
		F	N		597	1135	
		C			1988	896	

TABLE 1—(*Continued*)

Experiment	% Egg concentration	Sera used	Ave. % recovery of P^{32}	Cpm eggs	Cpm 100 mm.^{3} supernatant	Total cpm added to sample
7. *L.v.*	25	4 prime I	95	185	1157	6,938
		M_1 N		266	1195	
		C		3257	566	
8. *L.v.*	25	4 prime I*	91	138	796	4,944
		M_1 N*		148	776	
		C		262	784	
9. *L.v.*	25	a prime I	95	389	1423	9,495
		a N		968	1428	
		C		1198	1282	
10. *A.p.*	25	8 prime I	97	116	1104	12,922
		8 N		263	1436	
		C		652	1442	
11. *A.p.*	25	6 prime I	95	653	1488	9,900
		6 prime (0.45/1) I		1458	1454	
		6 N*		1282	1462	
		C		3360	835	

* Serum code:

4 prime—anti-*Lytechinus* fertilizin
10 prime—anti-*Lytechinus* fertilizin
a prime—anti-*Lytechinus* fertilizin plus unfertilized eggs
6 prime—anti-*Arbacia* fertilizin
8 prime—anti-*Arbacia* fertilizin
6 —corresponding pre-injection sera
8 —corresponding pre-injection sera
a —corresponding pre-injection sera
Th. —normal sera from 3 uninjected rabbits
F. —normal sera from 3 uninjected rabbits
M_1 —normal sera from 3 uninjected rabbits

in order to by-pass the lag and transition phases of phosphate uptake. The length of the lag period varies from female to female within a species (Whiteley and Chambers, 1960). These authors state that an average lag of 15 minutes in P^{32} uptake occurs following fertilization in *Strongylocentrotus purpuratus*. A count of 50 or 100 mm.^{3} of the P^{32}-sea water served to indicate total radioactivity at zero time. After incubating with slow rotation at 1 rpm for 60 minutes at 25° C., the vials were centrifuged and 100 mm.^{3}-aliquots of the supernatant taken. The eggs were washed three times in 2-ml. volumes of cold (0° C.) sea water, quantitatively transferred to planchets, and dried. Some difficulty was experienced in transfer of fertilized, demembranated eggs, in that these eggs sometimes tended to stick to glass. Percentage recovery of radioactivity was calculated after counting the dried samples with a thin-end-window Geiger-Muller tube (1.8 mg./cm.^{2}) within a low background (17 cpm) shield. The results are expressed as counts per minute per 0.25 ml. egg suspension, or per 100 mm.^{3} supernatant sea water. Experiments with 90% recovery or better are included. Comparisons made between experiments are of necessity qualitative since egg suspensions were prepared on the

Discussion

The failure of the antisera to activate the transport mechanism, as do other parthenogenic agents (Whiteley and Chambers, 1960), even though the respiratory rate in the presence of antisera against fertilizin is greatly increased, rules against activation of the eggs by such sera. Most probably, the increased respiration represents an increased oxygen consumption without accompanying oxidative phosphorylation, as with dinitrophenol or other agents (Krahl, 1950). Such uncoupling might be brought about by microscopically invisible cytolytic changes induced by the antisera (Brookbank, 1959).

The inhibition of phosphate uptake of fertilized eggs by antisera and other serum proteins may be ascribed to steric hindrance of transport, with antibody molecules establishing a more permanent bond with the egg surface than other proteins, as evidenced by centrifugation experiments (Tyler and Brookbank, 1956b). The inhibition by normal sera and BSA does mimic to some extent the inhibition of transport by p-chloromercuribenzoate (p-CMB) applied during the lag phase (Whiteley and Chambers, 1960). In concentration of 1.4×10^{-4} *M*, p-CMB virtually abolishes phosphate transport without inhibiting or delaying cleavage, a situation paralleled by normal sera and BSA. The effect of p-CMB is abolished by 10^{-3} *M* cysteine. The presence of at least some available cysteine residues in native proteins would seem to rule against anything similar in the mode of action of p-CMB and serum proteins. From the above information, it seems reasonable to conclude that: (1) phosphate uptake can be severely curtailed, without interfering with cleavage, by normal sera or BSA; (2) immune sera, diluted to the point where uptake approximates that of eggs in normal sera, continue to inhibit cleavage. Point (1) indicates a lack of dependence of early development on rapid uptake of phosphate. Point (2) would lead to the conclusion that cleavage inhibition by antisera against fertilizin does not result from decreased transport of phosphate.

Correlation of per cent inhibition of transport with nitrogen (protein) content of dialyzed sera or BSA is reasonably good only when BSA alone is considered, or when dilutions of a given serum are compared with one another (Table I, Experiment 11). In view of the complexity of mammalian sera and the lack of knowledge of the type of union between eggs and various serum proteins, it is not surprising that a more clear-cut relationship was not found. The possibility also exists of effects on P^{32} transport by non-dialyzable, non-nitrogenous serum compounds.

It is of interest that embryos in undiluted, sea-water-dialyzed, normal sera rarely, if ever, gastrulate. Lower concentrations of normal sera (0.5–1%) allow gastrulation but result in radially symmetrical larvae (Brookbank, unpublished data) with small skeletal spicules. In view of the lowered phosphate transport in the presence of serum proteins, one might postulate that lack of sufficient quantity of this ion ultimately may produce developmental abnormalities of primary mesenchyme metabolism and function during and after gastrulation.

Summary

1. Normal rabbit sera and antisera prepared in rabbits against sea urchin fertilizin were shown to have an inhibitory effect on uptake of phosphate by

fertilized eggs. Immune sera caused more pronounced decreases in phosphate transport.

2. Bovine serum albumin solutions caused similar decreases in rate of phosphate transport.

3. Inhibitory effects of proteins were presumed to be due to a combination of protein with the egg surface, rather than a binding of phosphate by proteins in solution.

4. Antisera against fertilizin were without effect on phosphate uptake by unfertilized eggs.

5. The results are discussed in light of other works on egg activation and phosphate uptake.

LITERATURE CITED

Ballentine, R., and J. R. Gregg, 1947. The use of potassium biiodate in iodometric determination of ammonia. *Anal. Chem.*, **19**: 281–283.

Boell, E. J., 1945. Techniques for estimation of total nitrogen in tissues and in tissue extracts. *Trans. Conn. Acad. Arts Sci.*, **36**: 429–441.

Brookbank, J. W., 1959. The respiration of unfertilized sea urchin eggs in the presence of antisera against fertilizin. *Biol. Bull.*, **116**: 217–225.

Harvey, E. B., 1956. The American Arbacia and Other Sea Urchins. Princeton University Press, Princeton, New Jersey.

Krahl, M. E., 1950. Metabolic activities and cleavage of the egg of the sea urchin, *Arbacia punctulata*. A review, 1932–1949. *Biol. Bull.*, **98**: 175–217.

Litchfield, J. B., and A. H. Whiteley, 1959. Studies on the mechanism of phosphate accumulation by sea urchin embryos. *Biol. Bull.*, **117**: 133–149.

Lowry, O. H., N. J. Rosenbrough, A. L. Farr and R. Endall, 1951. Protein measurement with the folin phenol reagent. *J. Biol. Chem.*, **193**: 265–275.

Palmer, L., 1937. The shedding reaction in *Arbacia punctulata*. *Physiol. Zoöl.*, **10**: 352–367.

Perlmann, P., 1957. Analysis of the surface structure of the sea urchin egg by means of antibodies. I. Comparative study of the effects of various antisera. *Exp. Cell Res.*, **13**: 365–390.

Perlmann, P., 1959. Immunochemical analysis of the surface of the sea urchin egg—An approach to the study of fertilization. *Experientia*, **15**: 41–52.

Tyler, A., 1949. A simple, non-injurious method for inducing spawning of sea urchins and sand dollars. *Coll. Net*, **19**: 19–20.

Tyler, A., 1959. Some immunobiological experiments on fertilization and early development. *Exp. Cell Res. (Suppl.)*, **7**: 183–199.

Tyler, A., 1963. The manipulations of macromolecular substances during fertilization and early development of animal eggs. *Amer. Zool.*, **3**: 109–126.

Tyler, A., and J. W. Brookbank, 1956a. Antisera that block cell division in developing sea urchin eggs. *Proc. Nat. Acad. Sci.*, **42**: 304–308.

Tyler, A., and J. W. Brookbank, 1956b. Inhibition of division and development of sea urchin eggs by antisera against fertilizin. *Proc. Nat. Acad. Sci.*, **42**: 308–313.

Tyler, A., A. Seaton and J. Signoret, 1961. Further analysis of antidevelopmental action of antisera against fertilizins and against other egg extracts of sea urchins. *Amer. Zool.*, **1**: 394.

Whiteley, A. H., and E. L. Chambers, 1960. The differentiation of a phosphate transport mechanism in the fertilized egg of the sea urchin. Symposium of germ cells and development. Institut Intern. d'Embryologie and Fondazione A. Baselli; pp. 387–401.